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A Novel Protein Complex Linking the δ2 Glutamate Receptor and Autophagy: Implications for Neurodegeneration in Lurcher Mice

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Summary

Autophagy is a pathway for bulk degradation of subcellular constituents that is hyperactivated in many neurodegenerative conditions. It has been considered a second form of programmed cell death. Death of cerebellar Purkinje cells in lurcher animals is due to a mutation in GluRδ2 that results in its constitutive activation. Here we have identified protein interactions between GluRδ2, a novel isoform of a PDZ domain-containing protein (nPIST) that binds to this receptor, and Beclin1. nPIST and Beclin1 can synergize to induce autophagy. GluRδ2^{Lc}, but not GluRδ2^{wt}, can also induce autophagy. Furthermore, dying lurcher Purkinje cells contain morphological hallmarks of autophagic death in vivo. These results provide strong evidence that a direct link exists between GluRδ2^{Lc} receptor and stimulation of the autophagic pathway in dying lurcher Purkinje cells.

Introduction

Over the past decade, the identification of mutations causing neurodegeneration in humans and in model systems, and the dissection of molecular pathways that can contribute to developmental or pathologic cell death in vivo and in vitro, have led to important advances in our understanding of neurodegenerative disease (Heintz and Zoghbi, 2000). These studies have focused a great deal of attention on pathways that contribute to death execution within neurons and on the significance of these processes to the pathophysiology of disease. For example, a role for apoptosis in both normally occurring developmental cell deaths and in certain aspects of pathologic neuronal loss in adult animals has been clearly documented (Vaux and Korsmeyer, 1999; Dirnagl et al., 1999). Recent evidence has suggested that autophagy may be a second program for death execution, since it is activated in parallel with apoptosis under a variety of conditions (Xue et al., 1999) and since constitutive activation of autophagy can lead to cell death (Schwartz et al., 1993; Dudek et al., 1997). Autophagy has been most extensively investigated as a pathway for bulk degradation of subcellular constituents in response to nutrient deprivation (Klionsky and Emr, 2000). It involves rearrangement of subcellular membranes into autophagosomes and autophagic vacuoles, which engulf cytoplasmic matrix and organelles and deliver them to lysosomes for degradation.

Genetic studies in yeast have revealed a complex pathway for the activation and execution of autophagy and have identified a large number of genes that are required for specific stages of this process (Ohsumi, 2001). The yeast Apg6 (Vps30p) gene functions in association with several other proteins to form a specific phosphatidylinositol-3-kinase complex that regulates autophagy (Kametaka et al., 1998). Beclin1, the human ortholog of yeast Apg6/Vps30p, can complement yeast Apg6 function in autophagy and promote autophagy when overexpressed in cultured human MCF7 cells (Liang et al., 1999). It is also found in association with phosphatidylinositol-3-kinase in mammalian cells, principally in association with the trans-Golgi network, where it is thought to be involved in the regulation of membrane dynamics during autophagy (Kihara et al., 2001). Beclin1 was first isolated in a screen for Bcl-2 binding proteins that might play a role in Bcl-2 dependent protection against Sindbis virus replication and induction of apoptosis in the brain (Liang et al., 1998). These studies identify Beclin1 as an important regulator of autophagy in mammalian cells and suggest it as a potential link between autophagy and apoptosis.

To identify pathways that contribute to neurodegeneration in vivo, we have pursued genetic and biochemical studies of the lurcher mutant mouse (Phillips, 1960). Heterozygous lurcher mice are ataxic due to degeneration of the cerebellar cortex during the first 4 postnatal weeks (Phillips, 1960; Caddy and Biscoe, 1975; Wilson, 1975). Constitutive activation of the GluRδ2^{Lc} causes an inward current and subsequent death of Purkinje cells that is cell autonomous (Wetts and Herrup, 1982) and analogous to glutamate-mediated toxicity in vivo and in vitro (Zuo et al., 1997; Heintz and Zoghbi, 2000). Secondary death of cerebellar granule cells and inferior olivary neurons follows Purkinje cell death due to loss of their afferent targets (Wetts and Herrup, 1982). Recent evidence has demonstrated that apoptotic pathways are activated in both Purkinje cells and granule cells in lurcher animals (Norman et al., 1995; Wullner et al., 1995; Selimi et al., 2000a). However, genetic tests of a requirement for Bax in lurcher-mediated neurodegeneration have demonstrated that the apoptotic pathway plays a minor role in the loss of Purkinje cells, although it is required for granule cell death in these animals (Doughty et al., 2000; Selimi et al., 2000b). Therefore, death of cerebellar Purkinje cells in response to constitutive GluRδ2^{Lc} activation must also involve pathways that are Bax independent.

GluRδ2 is specifically expressed in Purkinje cells in the cerebellum, where it is localized at the postsynaptic density (PSD) of parallel fiber synapses (Takayama et al., 1995; Landsend et al., 1997). Characterization of proteins present in the PSD and binding to the C terminus of NMDA and AMPA type glutamate receptors has led to several novel insights into mechanisms contributing to their function and regulation (Kennedy, 2000;

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Scannevin and Huganir, 2000; Sheng, 2001; Grant and O'Dell, 2001). These studies have provided a paradigm for the consideration of ionotropic glutamate receptors as multifunction proteins that are used in cells as ion channels that contribute to their electrical properties, and as cell surface receptors that organize signal transduction events within specific neuronal microdomains and control plasticity.

To gain insight into signaling pathways that may be associated with the GluRδ2 receptor and whose constitutive activation might contribute to Purkinje cell death in lurcher mice, we have screened for proteins that can specifically interact with the C terminus of GluRδ2. We report here that GluRδ2 is linked to the process of autophagy through novel protein-protein interactions involving a novel isoform of PIST (Neudauer et al., 2001) and Beclin1 (Liang et al., 1998) and that these proteins can synergize to induce autophagy in heterologous cells. Furthermore, we demonstrate that autophagy is activated by heterologous expression of the GluRδ2^{Lc} and in dying lurcher Purkinje cells in vivo. Since autophagy has been considered a second form of programmed cell death, our results provide evidence that constitutive activation of this pathway may contribute to Purkinje cell death in lurcher mice. Furthermore, they suggest that in human neurodegenerative diseases such as Huntington's disease (Sapp et al., 1997; Kegel et al., 2000; Petersen et al., 2001), Alzheimer's disease (Cataldo et al., 1996; Nixon et al., 2000), and Parkinson's disease (Anglade et al., 1997), the activation of autophagy might result from hyperactivation of neuronal cell surface receptors.

Results

Identification of a Novel Protein (nPIST) that Interacts with the C Terminus of GluRδ2

To identify signaling pathways that may be directly activated in response to the lurcher mutation, we have used the yeast two-hybrid system (Fields and Song, 1989) to screen for proteins interacting with the C-terminal cytosolic tail of GluRδ2. We obtained five independent clones encoding a novel PDZ domain-containing protein that strongly interacts with GluRδ2. Sequence analysis of the clones encoding the fusion proteins established that each isolate encoded a complete PDZ domain, suggesting that this domain might be involved in the interaction with GluRδ2. During the course of these studies, a protein (PIST) highly homologous to the protein that we had isolated was discovered to interact with the GTPase TC10 (Neudauer et al., 2001). Comparison of our sequence data with the published sequence of PIST indicated that the cDNAs we were studying encoded an additional eight amino acids at the beginning of the second coiled-coil domain (Figure 1A). To determine whether this isoform of PIST was differentially expressed, we performed RT-PCR assays to test for alternative mRNAs in a variety of tissues. As shown in Figure 1B, the PIST mRNA encoding the additional eight amino acids (nPIST) is highly enriched in whole brain and cerebellar mRNA. It contains three easily recognizable structural domains: two coiled-coil domains between amino acids 78 and 131, and 144 and 210, and a PDZ domain between amino acids 288 and 371.

To assess whether the expression of nPIST is regulated in a similar manner as GluRδ2 during cerebellar development, we analyzed the expression of both proteins during postnatal development. Western blot analysis of these proteins in total cerebellar extracts reproduced the observation that GluRδ2 expression is increased as the cerebellum matures (Takayama et al., 1995), and demonstrated that there is also an increase in nPIST expression during postnatal development (Figure 1C). Since GluRδ2 has been localized to the postsynaptic density (PSD) of Purkinje cell:parallel fiber synapses (Takayama et al., 1995; Landsend et al., 1997), we were next interested in determining whether nPIST might also be present at the PSD. nPIST cofractionated with GluRδ2 in cerebellar extracts and was enriched in both the synaptosomal and PSD fractions prepared from these extracts (Figure 1D). As expected, the abundant Purkinje cell cytoplasmic Ca^{2+} buffering protein calbindin did not cofractionate with nPIST and GluRδ2.

Given the coexpression and cofractionation data presented above, we next tested whether nPIST and the C terminus of GluRδ2 could interact in mammalian cells. Full-length N-terminal FLAG-tagged nPIST and an N-terminally FLAG-tagged fragment containing the last 155 amino acids of GluRδ2 were expressed in HEK293 cells. Extracts prepared from these cells were then immunoprecipitated with polyclonal antibodies to GluRδ2 and assayed for the presence of nPIST and the GluRδ2 C terminus by Western blot using anti-FLAG M2 monoclonal antibodies. As shown in Figure 2A (left panel), the FLAG-tagged nPIST and GluRδ2 C-terminal fragment were expressed well in HEK 293 cells when transfected individually or in combination. The anti-GluRδ2 polyclonal antiserum immunoprecipitated the FLAG-tagged GluRδ2 C-terminal fragments from these extracts and specifically coimmunoprecipitated nPIST when it was coexpressed with the GluRδ2 C-terminal fragment (right panel).

Previous studies have established that stable interactions can occur between the amino acids present at the extreme C terminus of glutamate receptors and the PDZ domains of several adaptor proteins, including PSD95 (Kornau et al., 1995; Niethammer et al., 1996), SAP102 (Muller et al., 1996), ABP (Srivastava et al., 1998), GRIP (Dong et al., 1997), and PICK1 (Xia et al., 1999). To determine whether the GluRδ2/nPIST interaction was similar, additional experiments to address the specificity and domain requirements for the GluRδ2/nPIST interaction were performed. As shown in Figure 2B, yeast two-hybrid studies established that nPIST can strongly interact with both GluRδ2 and its close relative GluRδ1 (Yamazaki et al., 1992; Lomeli et al., 1993). These interactions were either reduced or abolished if the final three amino acids of these receptors were deleted. No interaction was observed with the C terminus of GluR2, an AMPA receptor also present at the Purkinje cell:parallel fiber synapse (Hampson et al., 1992).

The fact that all nPIST clones obtained in the yeast two-hybrid screens contained an intact PDZ domain, taken together with studies demonstrating that PDZ domains can directly bind to the C terminus of cell surface receptors, strongly suggested that the nPIST PDZ domain would be required for interaction with GluRδ2. However, the interaction of PIST with the TC10 GTPase

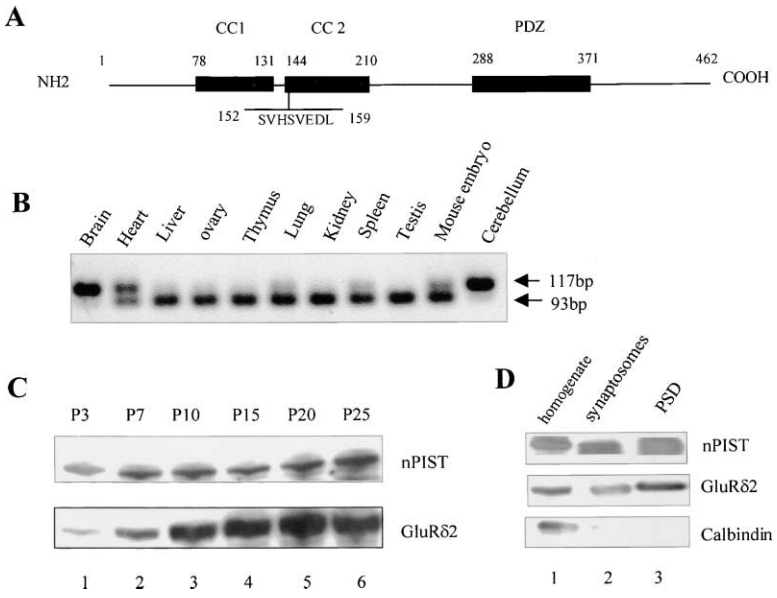

Figure 1. Identification of a PDZ-Containing Protein nPIST as a GluRδ2 C Terminus-Interacting Protein

(A) Schematic diagram of full-length brain-specific isoform nPIST protein identified from yeast two-hybrid screening. Solid boxes indicate the position of coiled-coil (CC1 and CC2) and PDZ domains. SVHSVEDL are the additional 8 amino acids specific for nPIST.

(B) RT-PCR products on total RNAs from different adult tissues and whole mouse embryo were separated on 2.5% agarose gel. The upper band contains 24 extra nucleotides encoding the 8 amino acids shown in (A).

(C) Developmental expression of nPIST in cerebellum. Homogenates (25 μg) of mouse cerebellum of indicated ages were probed with either anti-nPIST antibody or anti-GluRδ2 antibody.

(D) Immunoblots of subcellular fractions from cerebellum. Lane 1, homogenate (20 μg protein); lane 2, synaptosome fraction (4 μg protein); lane 3, PSD fraction (2 μg protein). nPIST, GluRδ2, and Calbindin were visualized by immunobloting with anti-nPIST antiserum (1:500), anti-GluRδ2 (1:500), and anti-Calbindin (1:500; Sigma) antibodies.

(Neudauer et al., 2001) involves a leucine-rich motif present in the second coiled-coil domain. Thus, to directly test domains in nPIST responsible for binding to GluRδ2 in mammalian cells, a series of constructs was prepared to specifically determine the requirement for known structural domains in this interaction (Figure 2C). Each of the nPIST constructs used for this assay included green fluorescent protein (GFP) fused at the N terminus so that both coimmunoprecipitation and double immunofluorescence colocalization studies could be performed. The GluRδ2 construct used for this analysis encoded the full-length GluRδ2 protein with a FLAG epitope fused to its mature amino terminus. As shown in Figure 2D, each of the GFP/nPIST fusion constructs containing an intact PDZ domain (GFPnPIST, GFP nPISTΔC, and GFPnPISTΔCC) was able to coimmunoprecipitate GluRδ2. In contrast, in cells expressing GFP or the GFP/nPIST fusion in which the PDZ domain had been deleted (GFPnPISTΔPDZ), precipitation with GFP antibodies was not able to bring down GluRδ2. To confirm these results, colocalization of the GFP/nPIST derivatives and GluRδ2 was assayed by direct visualization of the GFP/nPIST fusions and immunofluorescence detection of the FLAG-tagged GluRδ2. As shown in Figure 3, the colocalization results for GFPnPIST, GFP nPISTΔC, and GFPnPISTΔPDZ confirmed the results of the coimmunoprecipitation assays; GFPnPIST and GFPnPISTΔC colocalized with GluRδ2, whereas GFP nPISTΔPDZ did not. An unexpected result was obtained in the GFPnPISTΔCC experiments. In this case, colocalization with GluRδ2 was observed in the cytoplasm of cotransfected cells, but a large proportion of the GFPnPISTΔCC protein in these cells was present in the nucleus and did not colocalize with GluRδ2, indicating that sequences required for proper partitioning of nPIST between the nucleus and cytoplasm are present near or within the coiled-coil domain of nPIST. Taken together, our results demonstrate that the GluRδ2/nPIST interaction is specific and that it requires the final three amino acids at the C terminus of GluRδ2 and the nPIST PDZ domain.

Interaction of the nPIST Coiled-Coil Domain with Beclin1

The properties of nPIST and our evidence concerning its mode of binding to GluRδ2 strongly suggested that it might serve as an adaptor protein to organize signal transduction events in response to GluRδ2 activation. Given the high degree of conservation of coiled-coil domains between mouse nPIST and that of *C. elegans*, it seemed likely that these domains in nPIST could provide a scaffold for the assembly of other molecules that might play an important role in signaling through the GluRδ2 receptor. To address this issue, a second yeast two-hybrid screen was performed using the nPIST coiled-coil domains (residues 34–231; Figure 1A). In this screen, two independent isolates of the recently identified protein Beclin1 were obtained as strongly interacting partners for nPIST (residues 160–327, residues 201–372; Figure 4A). This was of great interest because Beclin1 was originally identified as a protein that might play a role in apoptosis through interaction with the BH1 domain of the Bcl-2 family proteins (Liang et al., 1998) and because it is the mammalian ortholog of the yeast Apg6/Vps30 gene, which plays an important role in autophagy (Kametaka et al., 1998).

To confirm the interaction between nPIST and Beclin1 revealed in the yeast two-hybrid screen, several assays were employed. First, GST pull-down assays were performed using GST-nPIST fusions containing the coiled-coil domain (residues 34–232) and myc-tagged Beclin1 (Beclin1-myc) from in vitro translation reactions. As shown in Figure 4B, beads carrying GST-nPIST were able to precipitate Beclin1-myc from the extract, whereas control GST beads were not able to bring down Beclin1-myc from the in vitro translation mixture. Coim-

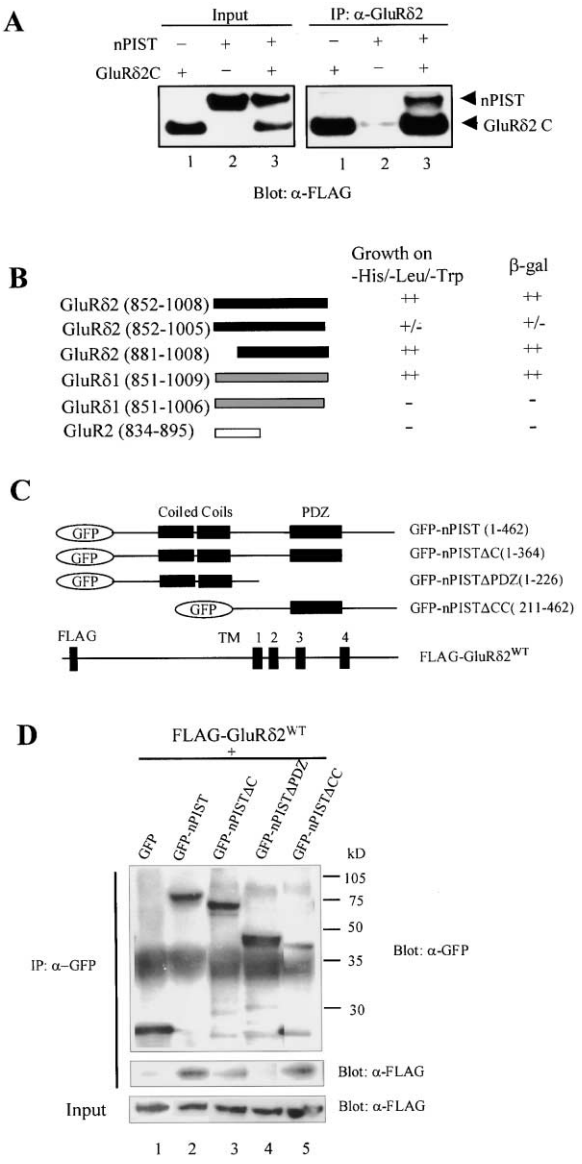

Figure 2. Specific Interaction between GluRδ2 and nPIST

(A) Extracts from HEK 293T cells transfected with pFLAG-GluRδ2 C terminus (GluRδ2C, lane1), pFLAG-nPIST (nPIST, lane 2), or combination of pFLAG-GluRδ2-C and pFLAG-nPIST (lane 3) were separated on SDS-PAGE and probed with anti-FLAG antibody. The expression of proteins from input extract is shown on the left panel; the complex pulled down by immunoprecipitation (IP) with anti-GluRδ2 antibody is on the right panel.

(B) Results from a yeast two-hybrid experiment using nPIST (residues 104–378, in pGAD10/pACT2) and C-terminal constructs of GluRδ2, GluRδ1, GluR2, and deletion mutants of GluRδ2 and GluRδ1 (pAS2-1 vector), as delineated in the schematic diagram. The interactions between nPIST and various constructs were determined by the ability of yeast colonies to grow in triple dropout media and by the induction of β-galactosidase activity.

(C) Schematic diagram showing GFP-nPIST or GFP-nPIST mutant fusion proteins and FLAG-tagged wild-type GluRδ2 (FLAG-GluRδ2^{WT}). The four transmembrane domains (TM) of GluRδ2 are indicated.

(D) Cotransfection of HEK-293T cell with FLAG-GluRδ2^{WT} and various GFP-fusion constructs as indicated in lanes 1–5. Transfected cells were lysed and immunoprecipitated (IP) with anti-GFP serum. Coprecipitates were analyzed by immunobloting using anti-FLAG antibody for GluRδ2, and anti-GFP antibody for GFP-fusion protein. Input cell lysates prior to IP were immunoblotted with anti-FLAG antibody for the presence of FLAG-GluRδ2^{wt} in all transfections.

munoprecipitation assays were also done to test the interaction of Beclin1-myc with a full-length nPIST FLAG epitope tagged at the N terminus (FLAGnPIST). Constructs expressing these proteins were cotransfected into HEK 293T cells in the appropriate combinations to test for this interaction. Anti-FLAG immunoaffinity beads were then used to precipitate FLAGnPIST, and the precipitates were assayed for coimmunoprecipitation of Beclin1-myc by Western blot using polyclonal antibodies raised against Beclin1 (Figure 4C). Beclin1 coprecipitated with FLAGnPIST in extracts expressing both proteins, whereas no Beclin1 was precipitated by the FLAG antisera from extracts prepared from cells expressing Beclin1 alone. These data demonstrate that FLAGnPIST and Beclin1-myc can form a complex in mammalian cells. Colocalization experiments were also done using direct fluorescence detection of GFPnPIST and immunofluorescence detection of Beclin1-myc by anti-myc antibodies staining (Figure 4D). GFPnPIST and Beclin1-myc colocalized in the transfected cells in the perinuclear area, consistent with reports that Beclin1 is present in the trans-Golgi network (Kihara et al., 2001). Since only the coiled-coil domain of nPIST was used in the yeast two-hybrid and pull-down assays, and since the two Beclin1 clones recovered from the two-hybrid assays both contained the Beclin1coiled-coil domains, we believe this interaction occurs by heterodimerization between the coiled-coil domains of these proteins.

Since both PIST and Beclin1 are widely expressed, we were next interested in their localization within the cerebellum and whether GluRδ2/nPIST/Beclin1 complexes exist in vivo. Polyclonal antibodies against nPIST and monoclonal Beclin1 antibodies were prepared, and they were checked for specificity using Western blots and immunofluorescence assays (see supplemental figures S1 and S2 online at http://www.neuron.org/cgi/content/full/35/5/921/DC1). As shown in Figure 4E, both nPIST and Beclin1 were present in cerebellar Purkinje cells, with significant labeling in both the cell bodies and dendrites (left panel). To directly address whether both nPIST and Beclin1 are present in a complex with GluRδ2 in vivo, immunoprecipitation assays were done using antibodies directed against GluRδ2 to isolate complexes containing the receptor and associated proteins from cerebellar extracts (right panel). nPIST and Beclin1 both coimmunoprecipitated with GluRδ2 from cerebellar extracts. Control experiments using preimmune serum did not result in nPIST or Beclin1 purification from these extracts. Taken together, these results confirm the yeast two-hybrid and heterologous cell expression experiments and also demonstrate that GluRδ2, nPIST, and Beclin1 form a complex in cerebellar Purkinje cells in vivo.

nPIST and Beclin1 Can Act Synergistically to Induce Autophagy

Beclin1 can rescue autophagy-deficient *apg6/vps30* yeast strains, and it can promote nutrient deprivation-

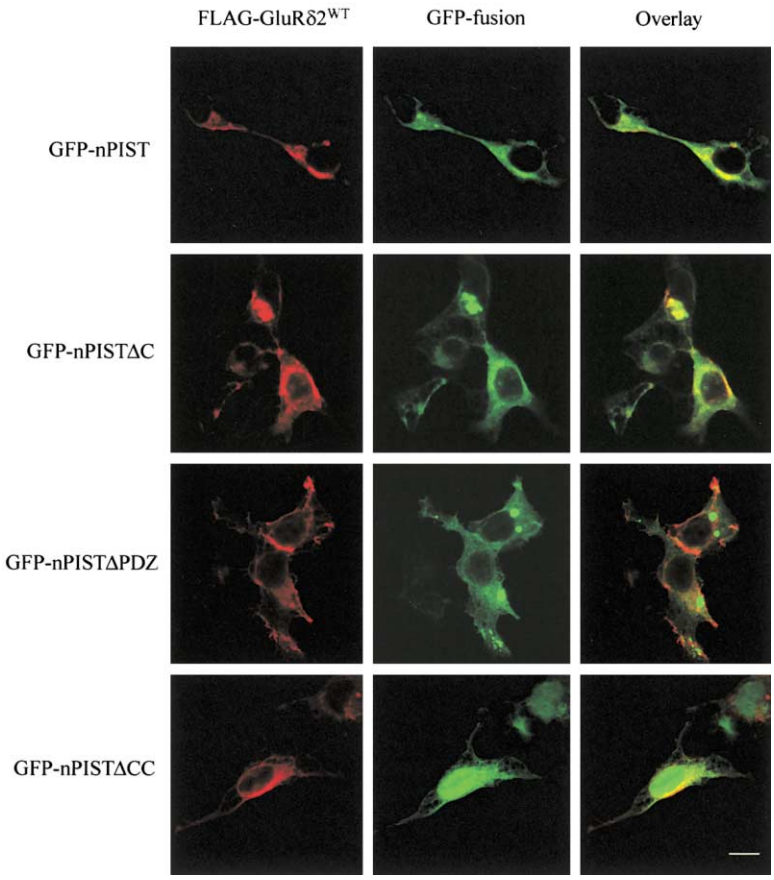

Figure 3. Colocalization of nPIST with GluRδ2 in Transfected HEK 293 Cells Depends on the PDZ Domain

The combination of plasmids used for transfection was described in Figure 2D. The distribution of FLAG-GluRδ2^{WT} in transfected cells is shown in red with anti-FLAG staining (Cy3), and GFP-fusion of nPIST or its mutants is shown as green. The overlay images are shown in the third column. Scale bar, 10 μm.

induced autophagy in MCF7 cells that cannot undergo an autophagic response to starvation in the absence of exogenously introduced Beclin1 (Liang et al., 1999). Beclin1 has also been shown to be present in a complex with phosphatidylinositol 3-kinase in the trans-Golgi network, where they may act together to control autophagy (Kihara et al., 2001). Given these activities of Beclin1, we were next interested in whether its role in autophagy could be modulated by nPIST. To establish a rapid assay for the activation of autophagy, we prepared a Beclin1-GFP fusion and used it to visualize autophagy in nutrient-deprived cultured cells. As shown in Figure 5A, the distribution of Beclin1-GFP in transfected cells was consistent with reports that native Beclin1 is localized to the perinuclear area, presumably the trans-Golgi (Kihara et al., 2001). In response to nutrient deprivation, there is a dramatic redistribution of Beclin1-GFP to large vesicular structures (Figure 5B). Quantitative analysis documented that approximately 5% of cells contained vesicular Beclin1-GFP staining under normal conditions, whereas more than 50% of the cells showed vesicular staining in this assay after starvation. To demonstrate that this reflects the activation of autophagy, cells were processed for electron microscopy and examined for ultrastructural evidence of autophagy and the localization of Beclin1-GFP. A large number of vesicular structures characteristic of autophagosomes and autolysosomes that are immunoperoxidase positive for Beclin1-GFP were observed in these cells (indicated with arrows) (Figures 5C and 5D). HRP-positive vacuoles wrapped with multiple layers of membranes, as frequently detected in cells undergoing autophagy, were noted (arrow head, Figure 5D). In some cases, rearrangement of membrane structure during the process of autophagosome formation was also observed and was positively stained by HRP (arrows, Figure 5E). These results establish that visualization of Beclin1-GFP fusion protein by fluorescence microscopy can be used as a simple and rapid assay for the activation of autophagy in mammalian cells.

It has been reported that Beclin1 can promote autophagy in yeast and in MCF7 breast epithelial cells (Liang et al., 1999). This is consistent with the observation that approximately 5% of cells transfected with Beclin1-GFP are undergoing autophagy under normal conditions (see above). Since GFP-nPIST and Beclin1 are colocalized in transfected cells under normal conditions (Figure 4D), we were next interested in whether nPIST could modulate the ability of Beclin1 to induce autophagy in HEK293 cells. As shown in Figure 6, in HEK293 cells cotransfected with full-length HA-nPIST and Beclin1-GFP, ~12% of cells exhibit the vesicular staining of Beclin1-GFP indicative of cells undergoing autophagy. This represents a slight increase over those not expressing nPIST, or a control expressing just the nPIST N-terminal 65 amino acids, indicating that under the conditions that we tested, coexpression of wild-type nPIST and Beclin1 were not sufficient to induce autophagy in the majority of cotransfected cells (Figure 6B). However, interaction of these proteins with GluRδ2 suggested to us that nPIST

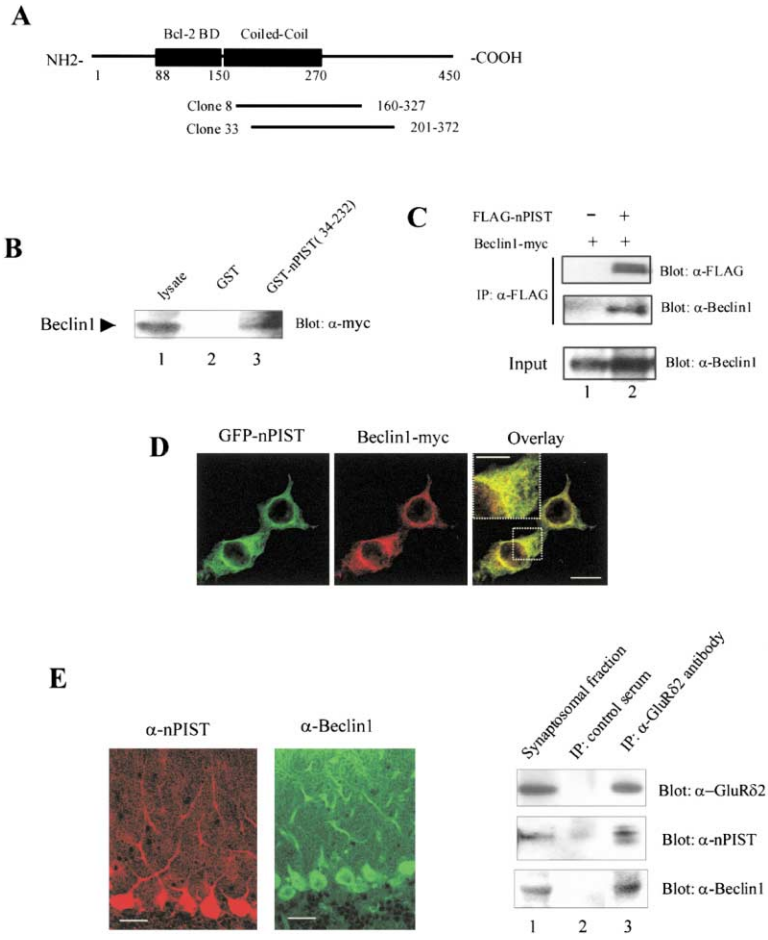

Figure 4. Interaction of the nPIST Coiled-Coil Domain with Beclin1

(A) Schematic diagram of full-length mouse Beclin1 protein containing Bcl-2 binding domain (Bcl-2 BD) and coiled-coil domain in black boxes. Two independent clones (8 and 33) isolated from yeast two-hybrid screening are shown at bottom with residues indicated.

(B) GST pull-down assay. Glutathione S-transferase (GST) beads coupled to purified GST or GST-nPIST (residues 34–232) fusion proteins were incubated with in vitro translated Beclin1. The amount of Beclin1 precipitated by either GST (lane 2) or GST-nPIST (34–232) (lane 3) was visualized by probing with anti-myc antibody. One-third of lysate used for pull down (lane 2 or 3) was loaded on lane 1.

(C) Cell extracts from HEK-293T cells transfected with Beclin1-myc alone (lane 1) or together with FLAG-nPIST (lane 2) were precipitated with anti-FLAG Sepharose. Immunoprecipitates were assayed on Western blots using either anti-Beclin1 polyclonal antibody (Novus Biologicals) or anti-FLAG antibody. The expression of Beclin1 in the input extracts is also shown.

(D) HEK-293 cells expressing Beclin1-myc and GFP-nPIST were stained with anti-myc antibody (in red). GFP-nPIST is shown in green. The overlay of both images is shown on the third panel. Scale bar, 10 μm. (High magnification of perinuclear area is shown in the square; scale bar, 5 μm.)

(E) Immunofluorescent staining of cerebellar slices with affinity-purified anti-nPIST antibody (in red) or monoclonal antibody against Beclin1 (in green), as visualized by confocal microscopy (scale bar, 40 μm). Right panel shows an immunopreciptitation of cerebellar synaptosomal fraction using GluRδ2 antibodies. Lane 1, 10 μg of input synaptosomal fraction; lane 2, IP with control serum; lane 3, IP with anti-GluRδ2 antibody. Proteins were detected using anti-nPIST serum, anti-Beclin1 polyclonal antibody, and anti-GluRδ2 antibody (Chemicon).

and Beclin1 activity might be regulated by interaction with other proteins through the nPIST PDZ domain. Therefore, we next tested whether a structural variant of nPIST lacking the PDZ domain might influence the activity of Beclin1 in the autophagic pathway. In cultures coexpressing an nPIST construct in which the PDZ domain was deleted (HA-nPISTΔPDZ) and Beclin1-GFP, vesicular Beclin1-GFP staining was observed in approximately 45% of the cotransfected cells (Figure 6B). In these cells, both Beclin1-GFP and HA-nPISTΔPDZ were found in vesicular structures (Figure 6A). If the cells were then placed under conditions of nutrient deprivation, a further increase in vesicular staining with Beclin1-GFP and HA-nPISTΔPDZ to approximately 55% of cotransfected cells was noted (Figure 6B). The appearance of these vesicles was very similar to those seen under normal culture conditions, although the number of vesicles per cell increased 4-fold under conditions of nutrient deprivation (data not shown). These results demonstrate that the interaction of nPIST with Beclin1 through its coiled-coil domains can modulate Beclin1 activity. They also suggest that interaction of nPIST with other proteins through its PDZ domain may regulate the activity of nPIST and Beclin1.

GluRδ2^{Lc}-Mediated Death in Heterologous Cells Involves Autophagy

We next asked whether the constitutive activation of GluRδ2^{Lc} could serve as a stimulus for the induction of autophagy and whether this process might contribute to GluRδ2^{Lc}-mediated cell death in vitro. Cells were cotransfected with constructs expressing GluRδ2 or GluRδ2^{Lc}, and Beclin1-GFP and were inspected by fluorescence microscopy. As shown in Figure 7A, cells expressing the constitutively activated GluRδ2^{Lc} glutamate receptor isoform contain the vesicular Beclin1-GFP-positive structures indicative of cells undergoing autophagy, whereas those expressing the wild-type GluRδ2 receptor typically do not. Cotransfection of GluRδ2^{Lc} with GFP confirmed the specificity of Beclin1-GFP targeting to these vesicular structures. Quantitation of these results (Figure 7B) established that expression of GluRδ2 results in a very slight increase of cells with vesicular Beclin1-GFP staining over those transfected with the control vector, whereas expression of the GluRδ2^{Lc} receptor resulted in a dramatic increase in Beclin1-GFP localization to vesicles, indicating a strong induction of autophagy in response to constitutive activation of this receptor. To assess whether autophagy might contribute to GluRδ2^{Lc}-mediated cell death, cells

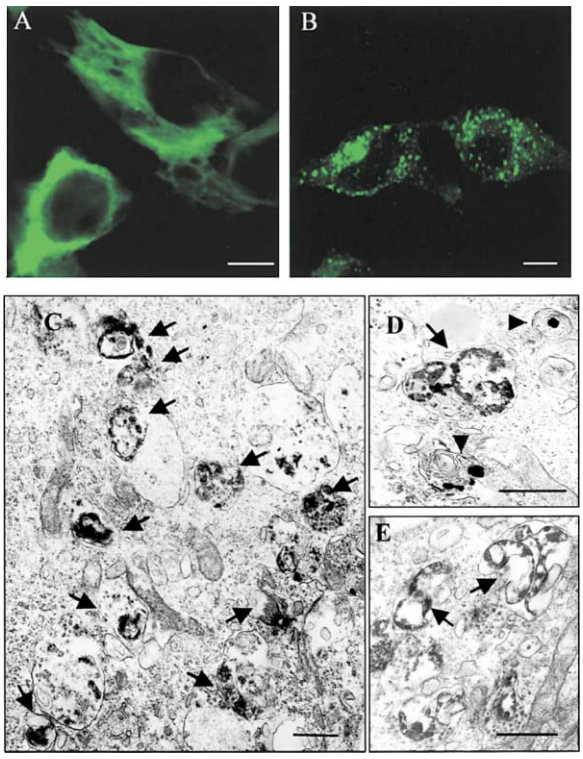

Figure 5. Redistribution of Beclin1-GFP in HEK 293 Cells after Induction of Autophagy by Nutrient Deprivation

(A and B) At 18–20 hr post transfection, HEK 293 cells transfected with Beclin1-GFP cDNA were washed with PBS and incubated in Earle's balanced salt solution (EBSS, Sigma) for an additional 1.5 hr. Transfected cells were not deprived (A) or deprived (B) of culture medium and examined by fluorescence microscopy. Scale bars, 10 μm.

(C–E) Electron microscopy study of HEK 293 cells transfected with Beclin1-GFP after nutrient deprivation. The sections were stained with anti-GFP antibody followed by labeling with HRP-conjugated secondary antibody. Electron dense peroxidase precipitates indicate localization of Beclin1-GFP fusion protein (arrows). Scale bars, 500 nm.

were again transfected with either the wild-type or mutant GluRδ2 receptor and assayed for death by staining with propidium iodide (Figure 7C). A substantial increase in dying cells was observed in the GluRδ2^{Lc}-transfected cells compared to cultures transfected with the wild-type receptor. Furthermore, death of GluRδ2^{Lc}-transfected cells was substantially decreased in the presence of 5 mM 3-methyladenine (3-MA), a potent inhibitor of autophagy (Seglen and Gordon, 1982). We conclude from this data that GluRδ2^{Lc} can activate autophagy and that autophagy can contribute to cell death as a consequence of GluRδ2^{Lc} activation.

Autophagy Is Activated in Dying Lurcher Purkinje Cells

The evidence we obtained in these studies strongly suggested that autophagy might be activated in dying lurcher Purkinje cells and that it might contribute to their death in lurcher mice. Electron microscopic studies of the cerebellum of lurcher mice have reported that lurcher Purkinje cells contain increased numbers of lysosomes, swollen and misshapen mitochondria, and other cytoplasmic structures that are not characteristic of wild-type Purkinje cells (Caddy and Biscoe, 1979; Caddy and Herrup, 1991; Dumesnil-Bousez and Sotelo, 1992). These early results are consistent with the activation of autophagy, although the presence of autophagosomes or autophagic vacuoles has not been reported. To establish whether autophagy is activated in dying lurcher Purkinje cells, additional electron microscopic studies were done. Vesicular structures bounded by two or more membranes, containing cytoplasmic ground substance or fragments of cellular organelles, were frequently observed in lurcher Purkinje cells (Figure 8). These structures are autophagosomes, and they clearly demonstrate that autophagy is activated in lurcher Purkinje cells. Autophagosomes were not observed in wild-type samples (data not shown). In some Purkinje cells in lurcher animals, very extensive vacuolization of the cytoplasm was evident, demonstrating the maturation of autophagosomes into autolysosomes in the final stages of autophagic cell death (Klionsky and Ohsumi, 1999; Bursch, 2001; Stromhaug and Klionsky, 2001). These data clearly demonstrate the activation of autophagic pathways in dying lurcher Purkinje cells and suggest that this process may contribute to lurcher-mediated cell death in vivo.

Discussion

The data we have presented establish four key points that are relevant to the function of GluRδ2 and the death of Purkinje cells in lurcher mice. First, they identify novel protein interactions between the C terminus of GluRδ2 and the PDZ domain of nPIST, and between the coiled-coil domains of nPIST and Beclin1. These interactions provide a physical linkage between the GluRδ2 receptor and Beclin1, a protein known to play an important role in autophagy (Liang et al., 1999). Second, they establish that in the absence of its PDZ domain, nPIST acts synergistically with Beclin1 to induce autophagy in heterologous cells, and that both of these proteins are present in autophagic vacuoles in cells undergoing autophagy in response to nutrient deprivation. Third, they demonstrate that expression of GluRδ2^{Lc}, but not wild-type GluRδ2, is able to induce autophagy. Fourth, they provide definitive morphologic evidence that an autophagic pathway is activated in dying Purkinje cells in the cerebella of lurcher mice. Autophagy is a pathway for lysosome-mediated bulk degradation of subcellular constitutents that has been considered a distinct form of programmed cell death (Xue et al., 1999; Bursch, 2001). Therefore, we propose that the stimulation of autophagy in response to the activation of GluRδ2^{Lc} receptor results from the novel properties of the GluRδ2/nPIST/Beclin1 complex. We suggest that release of Beclin1, or Beclin1 and nPIST, from the complex in response to GluRδ2^{Lc} is the crucial event required for activation of autophagy in lurcher Purkinje cells.

Apoptotic signaling is activated in lurcher Purkinje cells (Norman et al., 1995; Wullner et al., 1995; Selimi et al., 2000a). However, inhibition of the apoptotic pathway

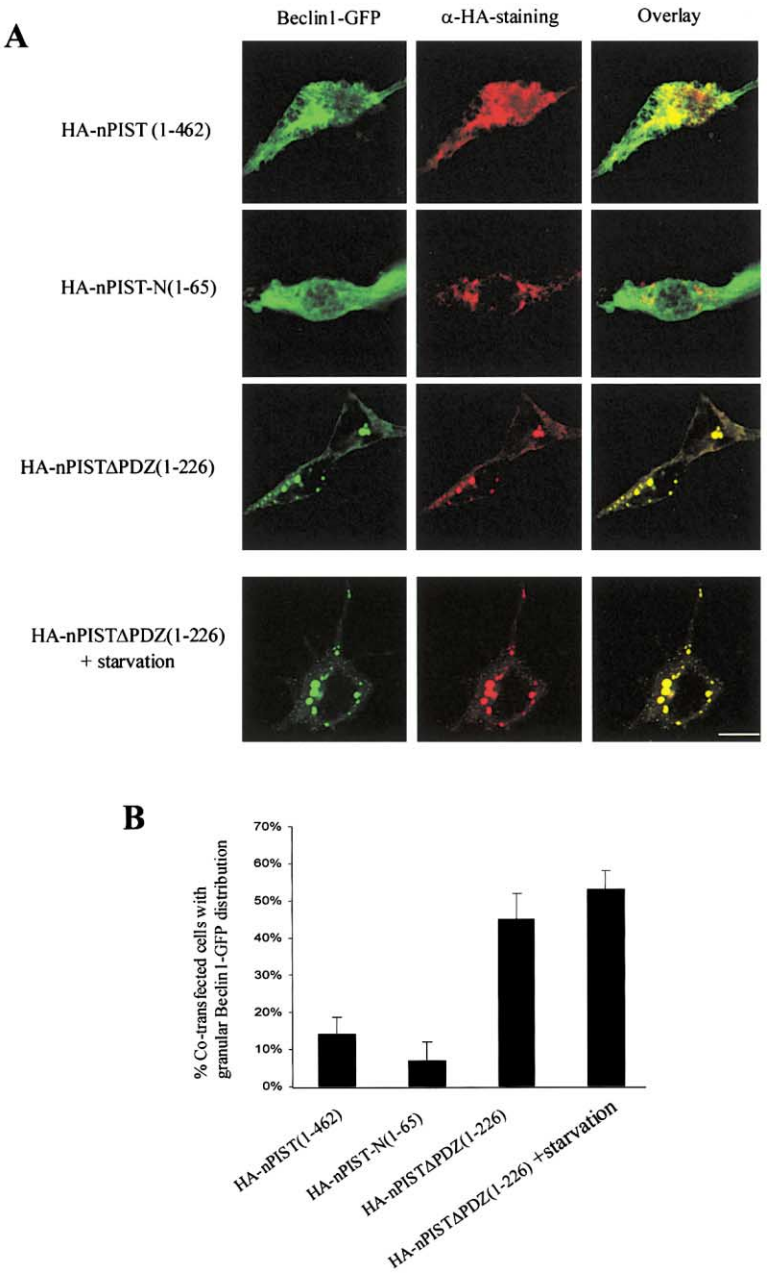

Figure 6. The Coiled-Coil Domains of nPIST and Beclin1 Act Synergistically to Induce Autophagy

(A) HEK-293T cells were cotransfected with plasmids encoding Beclin1-GFP and HA-tagged nPIST or nPIST deletion mutants. Transfected cells with or without nutrient deprivation were stained with anti-HA antibody (1:500; Clontech). HA-nPIST and its mutants are in red (Cy3), and Beclin1-GFP is in green. The overlay images are shown in the third column.

(B) Cells coexpressing both Beclin1-GFP and HA-tagged nPIST or indicated mutants and showing vesicular or granular distribution for Beclin1-GFP were scored from randomly chosen cells expressing both proteins. The results were collected from triplicate experiments. Values are mean percentage ± standard error.

by deletion of the Bax gene in lurcher mice is not sufficient to rescue Purkinje cell death (Doughty et al., 2000; Selimi et al., 2000b). These studies suggested that Purkinje cell death in lurcher mice must involve pathways that are Bax independent. The data presented here strongly support the hypothesis that autophagy is the Bax-independent pathway for lurcher Purkinje cell death that has been implicated from the genetic studies. Further support for this idea comes from the many studies that have reported overlap between apoptosis and autophagy. For example, Xue et al. (1999) have shown that NGF deprivation from cultured sympathetic neurons results in activation of both apoptotic and autophagic responses. They demonstrated that inhibition of autophagy with 3-MA results in delayed apoptotic death, and inhibition of apoptosis with pan-caspase inhibitors blocks morphologic apoptosis but does not prevent death. Based on these results, the authors suggest that autophagy and apoptosis are often activated by similar stimuli in neurons and that these pathways can be considered alternative mechanisms of death execution. Although no evidence to address this point has been presented in this study, reports that Beclin1 can also interact with Bcl-2 (Liang et al., 1998) are provocative in this regard. If Beclin1 can associate with Bcl-2 or other members of this family in vivo, then tethering of a complex containing nPIST, Beclin1, and a Bcl-2 family member to GluRδ2 would provide a biochemical mechanism for coordinate regulation of apoptotic and autophagic responses in lurcher Purkinje cells.

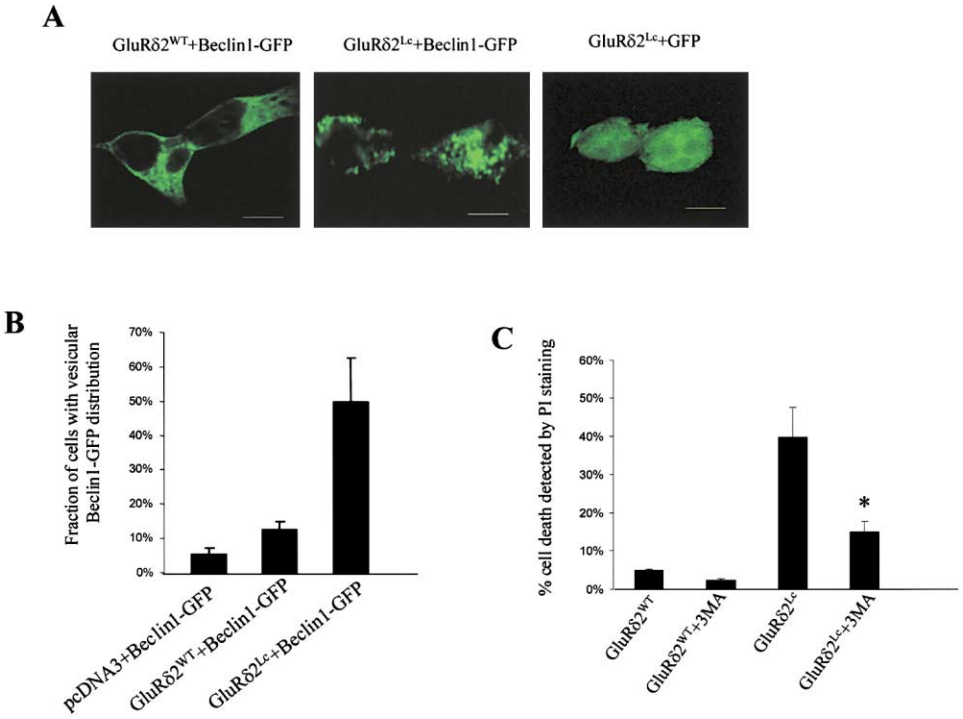

Figure 7. GluRδ2^{Lc}-Mediated Cell Death in HEK 293 Cells Involves Autophagy

(A) HEK-293T cells cotransfected with indicated plasmids were fixed at 24 hr post transfection and imaged using confocal microscopy.
(B) The number of transfected cells with punctate or granular distribution of Beclin1-GFP was scored against total number of randomly chosen cells expressing Beclin1-GFP. The results were collected from triplicate experiments. Values are mean percentage ± standard error.
(C) HEK-293T cells transfected with either FLAG-GluRδ2^{WT} or FLAG-GluRδ2^{Lc} incubated with 5 mM 3-methyladenine (3 MA) at 5 hr post transfection for a period of 18 hr. Cells were then stained live with 1 μg/ml propidium iodide (PI) for 5 min at 37°C and washed with PBS. Cells were detached from the culture dish and scored by fluorescence-activated cell sorting. (Miura and Yuan, 2000). The experiments were done in triplicate or quadruplicate. Values are mean percentage of dead cells ± standard error. *$p < 0.001$.

The identification and functional characterization of the GluRδ2/nPIST/Beclin1 complex and the activation of autophagy in lurcher Purkinje cells in vivo presented herein have important implications regarding recent reports of the activation of autophagy in several of the major human neurodegenerative disorders, including Alzheimer's disease (Cataldo et al., 1996), Parkinson's disease (Anglade et al., 1997), and Huntington's disease (Sapp et al., 1997; Petersen et al., 2001). Given our demonstration that nPIST can also interact with GluRδ1, which is widely expressed in the CNS, and the expression of nPIST and Beclin1 at many sites within and outside of the nervous system that do not express GluRδ1 or GluRδ2, it seems likely that the nPIST/Beclin1 complex may function in collaboration with other cell surface receptors and external signals. If this is the case, we anticipate that receptor-mediated activation of Beclin1 and its stimulation of autophagic pathways may also play an important role in human neurodegenerative disorders. There is, of course, no reason that intracellular mechanisms regulating nPIST and Beclin1 function might not also contribute to the activation of autophagy in these diseases. Given these considerations, it will be important to establish which other neuronal cell surface receptors are coupled to the nPIST/Beclin1 pathway and which intracellular regulatory events can modulate the activities of these proteins. For example, it has been reported recently that there is a dramatic increase in cytoplasmic vacuoles in Purkinje cells of SCA1 transgenic mice (Skinner et al., 2001). Although no evidence identifying these structures as autophagic vacuoles was presented, these observations are intriguing in light of observations that autophagy is activated in degenerating cells in Huntington's disease (Sapp et al., 1997; Petersen et al., 2001). While it seems improbable that activation of this pathway is a primary pathogenic event in triplet repeat disorders, it is quite possible that activation of Beclin1 and subsequent autophagic responses might be important secondary events that could contribute to pathogenesis.

The identification of the GluRδ2/nPIST/Beclin1 complex, and the activation of autophagy by the GluRδ2^{Lc} receptor in vivo and in heterologous cells, also has important implications for consideration of the functions of wild-type GluRδ2. Under extreme conditions, such as nutrient starvation or constitutive activation of the GluRδ2^{Lc} receptor, autophagy is a process by which cells digest themselves from within, eventually leading to death. Autophagy is a highly regulated vesicular pathway for delivery of cellular proteins, membranes, and organelles to lysosomes for degradation and recycling. It is involved in cellular remodeling in a variety of systems, including diurnal remodeling of photoreceptor cells in the mammalian retina (Reme and Sulser, 1977), filamentous differentiation of fungi (Cutler et al., 2001), and wing and eye development in flies (Thumm and

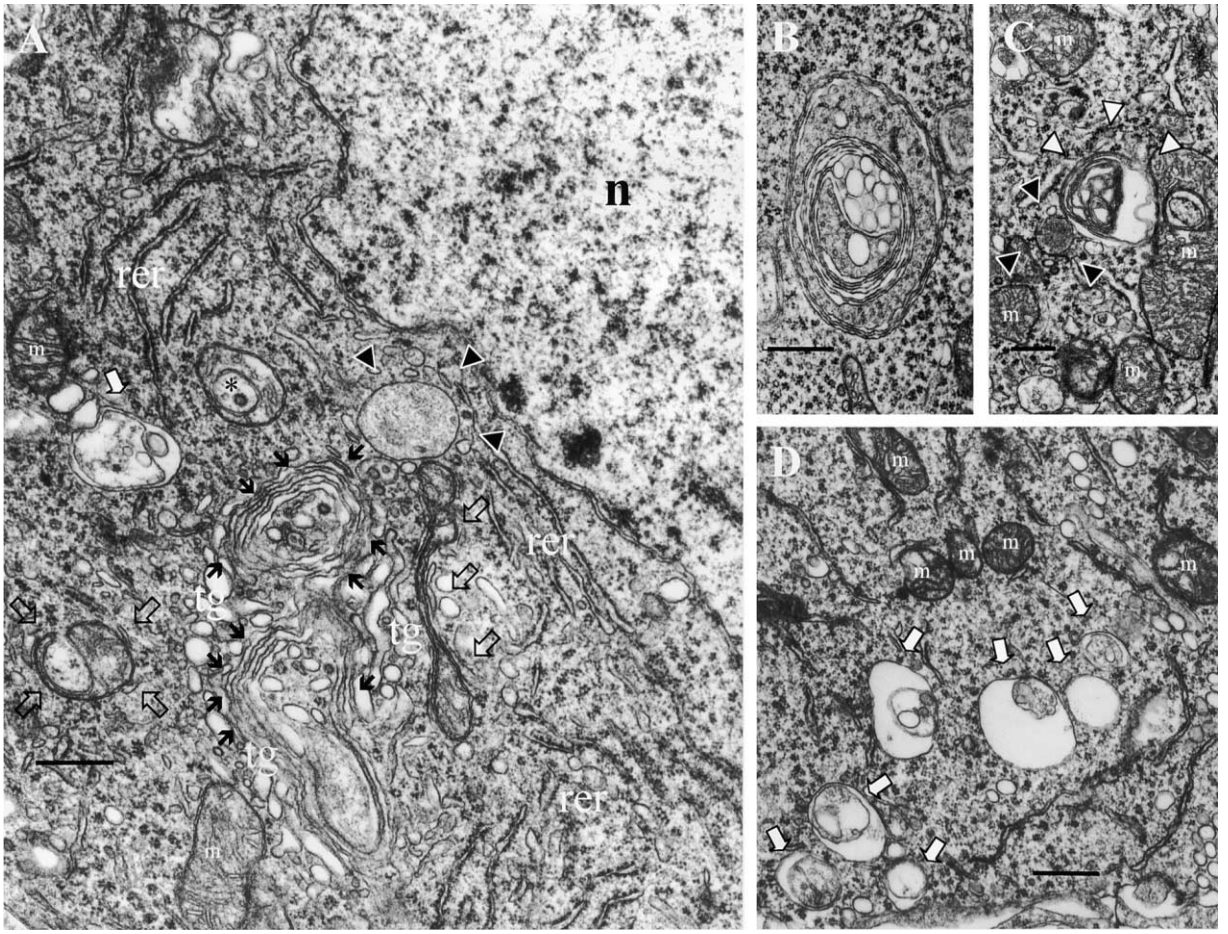

Figure 8. Autophagy in Lurcher Purkinje Cells

Four representative Purkinje cells from two different lurcher mice illustrate the presence of several autophagic profiles.

(A) Low magnification electron micrograph showing numerous profiles typical of autophagy detected at different stages of development. Membranes from the rough endoplasmic reticulum sequester cytosolic components in an early stage of autophagosome formation (open arrows). This process will lead to the formation of the characteristic double membrane vesicles, autophagosomes (asterisk). Autophagosomes wrapped by whorls of membranous material (small arrows) envelope cytoplasm and organelles. An autolysosome (white arrow), with a characteristic single membrane, is recognizable in the left hand portion of the picture.

(B) Autophagic vacuole with several double membranes enclosing cytosol and free ribosomes. These structures are common in the soma of degenerating Purkinje cells.

(C) Autophagosome (white arrowheads) contains residues of membranes and a large cysterna. Immediately adjacent to this is a lysosome (black arrowheads), perhaps in the initial stage of fusion with the autophagosome.

(D) Several autolysosomes (white arrows) in a Purkinje cell during the final stage of degeneration. The majority of these autolysosomes appear as sizable vacuoles bounded by a single membrane and containing only a small inner vesicle, where the residues of the lytic process remain. Scale bar, 500 nm; m, mitochondrion; tg, trans-Golgi; n, nucleus; rer, rough endoplasmic reticulum.

Kadowaki, 2001). Furthermore, PIST has recently been independently isolated in a yeast two-hybrid screen for proteins interacting with the C terminus of the cystic fibrosis transmembrane regulator (CFTR) and has been shown to modulate expression of CFTR on the plasma membrane (Cheng et al., 2002). In this case, PIST both decreased the rate of CFTR insertion into the plasma membrane and decreased its half-life in the plasma membrane. The mechanism for this role of PIST in trafficking of the CFTR receptor is not known. Although no evidence that Beclin1 or autophagic pathways are involved in CFTR trafficking has been obtained, the fact that activation of autophagy results in budding of vesicles from both the Golgi and the plasma membrane would be consistent with a role for this pathway in both trafficking of receptors to the cell surface and recycling of proteins from it. Given these considerations, it will be of interest to determine whether regulation of the GluRδ2/nPIST/Beclin1 complex in Purkinje cells is important for maintenance of proteins associated with the postsynaptic density at Purkinje cell:parallel fiber synapses. Such a role would be consistent with the failure of synapse stabilization and function that has been observed in GluRδ2 null mutant mice (Kashiwabuchi et al., 1995).

Delineation of the precise mechanism by which GluRδ2 activation leads to the induction of autophagy will require additional studies aimed at determining the properties of nPIST and Beclin1 in wild-type and lurcher Purkinje cells. While we propose that it is the release of

Beclin1, or nPIST and Beclin1, from the GluRδ2/nPIST/Beclin1 complex that is the crucial stimulus for the activation of autophagy in response to GluRδ2^{Lc}, there are several issues that must be addressed to obtain a detailed understanding of this process. First, we would like to know whether nPIST and/or Beclin1 are covalently modified in response to signaling through the receptor and whether this leads to release of one or both of these proteins from the complex. Second, if a protein kinase is an important component of the GluRδ2 signaling complex, it will be important to know the identity of the kinase and whether it is activated in response to ion flux through the receptor. Third, given the published data documenting an interaction between Beclin1 and Bcl-2, it would also be informative to know if any Bcl-2 family proteins are present in the complex and whether these proteins contribute to the induction of apoptotic events in lurcher Purkinje cells. Further investigation of these issues in lurcher mice and in heterologous cell systems will be required to explore these issues.

Experimental Procedures

Yeast Two-Hybrid Screening

Yeast two-hybrid screens were carried out using the MATCHMAKER II kit from Clontech, according to the manufacturers instructions. Bait constructs for the GluRδ2 C-terminal domain interacting protein screen contained residues 852–1008 of GluRδ2. ~10^6 clones from an adult brain library were screened, yielding five independent nPIST clones. Full-length nPIST was cloned by RT-PCR and RACE. PCR primers used to amplify the spliced variants (Figure 1B) are 5′-GTT GACTCTGGGGCCATCAAG-3′ and 5′-CACTTCAGCTTCAAGTCG AGC-3′. Bait constructs for nPIST binding proteins contained its coiled-coil domains (residues 34–231) and were used to screen ~2×10^6 clones, yielding two independent clones containing coiled-coil domains of Beclin1.

Constructs

The cDNA encoding mouse nPIST was cloned into pFLAG-CMV vector (Eastman Kodak) and was epitope-tagged with FLAG at its N terminus. GFP-fusions of nPIST and its mutants were prepared by PCR and subcloned into pEGFP-C1 vector (Clontech). The expression plasmids containing cDNA of GluRδ2 receptor wild-type and lurcher mutant were FLAG-tagged at the N terminus immediately after signal peptide sequence using pFLAG-CMV as vector. The C-terminal domain of GluRδ2 containing transmembrane domain IV (TM4) and intracellular domain fused with N-terminal FLAG following GluRδ2 signal peptide sequence was also subcloned into pFLAG-CMV vector. The cDNA containing mouse Beclin1 was inserted in-frame with myc-epitope tag at C terminus into pcDNA3-myc-His vector (Invitrogen). The EGFP/Beclin1 fusion was constructed using pEGFP-N3 (Clontech). N-terminal HA epitope-tagged nPIST constructs were prepared by subcloning PCR products into pVAX1 (Invitrogen). All constructs were confirmed by sequence analysis.

Antibodies

Antiserum against nPIST was raised from injection of rabbits with purified His-tagged coiled-coil domains (34–232) of nPIST (Cocalico Biologicals). Anti-nPIST antibody was purified using an affinity column prepared with purified His-tagged coiled-coil domain cross-linked to activated Sepharose 4B (Pharmacia). The monoclonal antibody against Beclin1 was generated by injection of mice (Green Mountain Antibodies, Burlington, VT) with purified His-tagged domain (1–163) expressed from pET28c vector. Ascite fluid was purified on a ProA column to a final concentration of 8 mg/ml.

Cell Culture, Transfection, and Immunoprecipitation

HEK 293T cells were maintained in 10% fetal bovine serum containing DMEM. Cells were transfected by standard calcium phosphate precipitation with a combination of expressing plasmids. At 48 hr post transfection, cells were washed with PBS and harvested in lysis buffer (25 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). The insoluble fraction was excluded by centrifugation at 14,000 × g for 15 min. The soluble fraction was precleared by incubating with protein A sepharose and collected after centrifugation. Immunoprecipitation was performed with 2 μg/sample of anti-FLAG sepharose (Sigma), 1 μg/sample of anti-GFP antibody (kind gift by Dr. M. Rout, The Rockefeller University), or 1 μg of anti-GluRδ2 antibody (Chemicon) incubating with precleared lysate at 4°C over night. Precipitates were washed five times with lysis buffer and eluted with reducing sample buffer.

Extract Preparation, Fractionation, and In Vivo Immunoprecipitation

Synaptosomal and PSD fractions were prepared as previously described (Carlin et al., 1980). Ten adult mouse cerebella were homogenized in buffer A (0.32 M sucrose, 25 mM HEPES, 1 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail) and centrifuged at 1,400 × g. The supernatant was further centrifuged at 14,000 × g for 15 min. The resulting pellet was resuspended in buffer A and loaded on a 0.85 M, 1.0 M, and 1.2 M sucrose step gradient followed by 82,500 × g centrifugation for 1 hr. The synaptosomal fraction was collected between 1.0 M and 1.2 M interface, diluted with equal volume of buffer B (1% Triton X-100 in 320 mM sucrose-HEPES [pH 7.4]), and incubated for 20 min at 4°C. This fraction was centrifuged at 32,800 × g for 30 min, and pellet resuspended in buffer A and reloaded on a 1.0 M, 1.5 M, and 2.0 M sucrose gradient. After centrifugation at 201,800 × g for 2 hr, the fraction between 1.5 M and 2.0 M was collected and mixed with equal volume of buffer B. The mixture was then centrifuged at 201,800 × g. The resulting pellet consists of the PSD fraction.

For immunoprecipitation from cerebellar extracts, 150 μg of the synaptosomal fraction was incubated in a buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM DTT, 1% NP-40, 0.5% DOC, and protease inhibitor cocktail. The lysate was clarified by centrifuging at 14,000 × g for 10 min. The supernatant was incubated with protein A beads cross-linked with either αGluRδ2 antibody or purified rabbit IgG from control serum according to the protocol "Seize X Protein A immunoprecipitation kit" (Pierce). After four washes, the beads were eluted with elution buffer provided by the kit plus 0.5% SDS and 1 mM DTT. The eluted fractions were resuspended in reducing sample buffer, boiled, and loaded on SDS-PAGE for immunoblotting analysis.

Immunofluorescence

Localization of nPIST and Beclin1 on cerebellar sections was carried out using monoclonal antibody against Beclin1 and affinity-purified polyclonal antibody against nPIST (see above). P30 C57/Bl6 mice were perfused and then postfixed with 4% paraformadehyde for 1 hr. Dissected cerebella were sectioned at 50 μm on a freezing stage microtome (Leica). Cerebellar slices were first blocked with PBS containing 0.05% Triton X-100 and 10% goat serum and then incubated with purified anti-nPIST antibody or monoclonal antibody against Beclin1 at 4°C overnight. The slices were washed and incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson) or Alexa488-conjugated goat anti-mouse IgG (Microprobes) for 45 min at RT. The sections were washed extensively, mounted, and examined using a Zeiss confocal microscope.

For localization in cultured cells, cultures were grown on poly D-lysine-coated coverslips to ~50% confluence. Cells were fixed with 4% paraformadehyde for 10 min at 18–20 hr post transfection. Primary anti-FLAG (M2) (Sigma; dilution 1:1000), anti-myc (Invitrogen; dilution 1:1000), or anti-HA (Invitrogen; dilution 1:500) antibodies were used to detect epitope-tagged proteins. Anti-mouse or anti-rabbit IgG Cy3-conjugated (Jackson) secondary antibodies were used at a 1:600 dilution.

Electron Microscopy

Wild-type and lurcher (P10) mice were intracardially perfused with 2.5% glutaradehyde in PBS, and the brains were dissected and postfixed overnight. After rinsing with 0.1% cacodylate on ice, cere-

bellar folia were isolated and postfixed with 1% osmium tetroxide in cacodylate on ice. The specimens were then treated en bloc with uranyl acetate and dehydrated with alcohol and propylene oxide, and embedded in Ducopan. 60 nm sections were collected and stained with uranyl acetate and lead citrate and examined. For immunoelectron microscopic analysis of Beclin1-transfected HEK 293 cells, cells were rinsed with PBS and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 100 mM Cacodylate buffer (pH 7.4) for 30 min on ice. After rinsing, the specimens were quenched with 50 mM ammonium acetate in buffer (30 min), treated with 0.5% Triton-X 100 in PBS (5 min), rinsed in PBS, blocked with 1% BSA in PBS (30 min), and incubated overnight at 4°C with anti-GFP antiserum (1:2000) in 0.1% BSA in PBS. Samples were washed and incubated with HRP-conjugated goat anti-rabbit-IgG (1:500) in 0.1% BSA-PBS overnight at 4°C. Coverslips were refixed in 0.1% glutaraldehyde and incubated in DAB solution (5 mg 3,3′ diaminobenzidine in 0.05 M Tris-HCl buffer [pH 7.2] containing 0.01% hydrogen peroxide) to visualize peroxidase. The coverslip was washed, fixed in 2.5% glutaraldehyde in 0.1 cacodylate (pH 7.4), post fixed in 1% osmium tetroxide, treated with 1% aqueous uranyl acetate, dehydrated in alcohol, and infiltrated with Epon. A Beem capsule filled with Epon was inverted over the monolayer on the coverslip in order for parallel sectioning to the substrate. Pale gold ultrathin sections were collected on Formvar carbon-coated copper grids. In both experiments, sections were examined and photographed under a JEOL 100CX electron microscope operated at 80 kV.

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